Mitochondrial STAT3: Powering up a potent factor.

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Abstract

The JAK-STAT3 signaling pathway is engaged by many cytokines and growth factor stimuli to control diverse biological processes including proliferation, angiogenesis, survival, immune modulation, and metabolism. For over two decades it has been accepted that STAT3-dependent biology is due to its potency as a transcription factor capable of regulating the expression of many hundreds of genes. However, recent evidence of non-canonical and non-genomic activities of STAT3 has emerged. The most exciting of these activities is its capacity to translocate into the mitochondria where it regulates the activity of the electron transport chain and the opening of the mitochondrial permeability transition pore. These have broad consequences including cell survival and the production of reactive oxygen species and ATP in both normal tissue and under pathological conditions. Despite these fascinating observations there are many key unanswered questions about the mechanism of STAT mitochondrial activity.

Key words

STAT3, mitochondria, electron transport, cancer, ischaemia/reperfusion, metabolism

Abbreviations

EGF, Epidermal Growth Factor; ETC, Electron Transport Chain; GAS, γ -Activated Sequence; I κ B, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor; JAK, Janus Kinase; LIF, Leukemia Inhibitory Factor; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; mPTP, mitochondrial Permeability Transition Pore; PDGF, Platelet-Derived Growth Factor; SOCS, Suppressor of Cytokine Signaling; STAT, Signal Transducer and Activator of Transcription; TIM, Transport Inner Membrane; TOM, Transport Outer Membrane.

Personal statement of my research.

My research interest centers on mechanisms of JAK-STAT signaling that are aberrantly activated to drive tumorigenesis. In particular my research has focused on understanding signaling mechanisms that co-operate with JAK-STAT pathways and non-canonical JAK-STAT activities. This research has contributed to the identification of a mitochondrial pool of STAT3 that augments the activity of the electron transport chain which is required for tumors driven by the Ras oncogenes. My current research program is focused on uncovering the mechanisms by which STAT3 is imported into the mitochondria and its activities within the mitochondria.

1. Introduction:

Inter- and intracellular communication is essential to the correct functioning of all organisms. This process is regulated by tightly controlled, complex signaling networks. One of the most potent families of signaling molecules are the *Signal Transducer and Activator of Transcription* (STAT) proteins. There are 7 members of this family, STAT1, 2, 3, 4, 5a, 5b and 6. STAT3 is required to transmit signals from a diverse array of cytokines and growth factors (including IL-6 family, IL-10, EGF, PDGF, LIF) and is essential for life [1]. The prevailing dogma of STAT3 signaling is that it exists as a latent, un-phosphorylated monomer in the cytosol until cytokines or growth factors engage their cognate receptor. Ligand/receptor interaction causes a change in conformation and concurrent activation of receptor associated Janus Kinase (JAK) proteins. Activated JAKs trans-phosphorylate each other and the cytoplasmic tail of the receptor on tyrosine residues that provides docking sites for STAT proteins to be recruited via their SH2 domain. Once recruited, STAT proteins are phosphorylated on a single carboxy-terminal tyrosine residue (Y705 in STAT3) by JAKs. This tyrosine phosphorylation alters the conformation of STAT dimers, which translocate into the nucleus and bind to consensus DNA sequences (γ -activated sequence (GAS) TTCNNNGAA) and initiate transcription. This pathway is tightly regulated and transient. STAT proteins can be dephosphorylated by protein-tyrosine phosphatases or STAT signaling can be impeded by the Suppressors of Cytokine Signaling (SOCS) proteins. The *SOCS* genes are direct transcriptional targets of STATs and they either block STAT-receptor recruitment or degrade target proteins through an ubiquitin directed mechanism.

2. Non-canonical STAT3 activities

The importance of JAK-STAT signaling has been revealed by knockout mice and by humans with spontaneous mutations in receptors, kinases e.g., JAK (severe combined immunodeficiency [2] and myeloproliferative disease [3]) or in STAT3 itself (loss of function mutations in hyper IgE syndrome [4]). The diversity of STAT3-dependent biological responses has, until recently, been considered to be entirely dependent on its activity as a tyrosine phosphorylated transcription factor. However, recent evidence suggests that there is far more complexity.

2.1 Transcriptional activity of unphosphorylated STAT3

Recombinant, unphosphorylated STAT3 binds to a DNA probe containing the STAT3 consensus binding site from the *c-fos* promoter, albeit with lower affinity than the Y705 phosphorylated form of STAT3 [5]. These data show that Y705 phosphorylation is dispensable for dimerization and DNA binding and possibly transcription of STAT3 target genes. Indeed, the reconstitution of STAT3 low or null cells with either wild-type or a STAT3 mutant incapable of phosphorylation on Y705 (Y705F) results in significant changes in the expression of similar numbers of genes. Interestingly, two thirds of these genes are unique to the specific phosphoform of STAT3 [6], suggesting that the transcriptional profile and likely the biological response will depend on the dominant STAT3 post-translational modification. These data imply that nuclear import of STAT3 is independent of Y705 phosphorylation and dimerization or that STAT3 can be chaperoned into the nucleus. Both are the case. Nuclear shuttling of wild type and Y705F mutant STAT3 are equivalent [7], suggesting that tyrosine phosphorylation has no impact on nuclear import of STAT3. Additionally, unphosphorylated STAT3 competes with I κ B for binding to the p65 and p50 subunits of NF- κ B. These STAT3-NF- κ B complexes enter the nucleus and bind to promoters containing both GAS and NF- κ B motifs (e.g in the *RANTES* gene) [8]. The transcriptional activity of unphosphorylated STAT3 is influenced by acetylation on K685 [9], however it is not clear whether this is dependent on NF- κ B.

The contribution of K685 acetylated STAT3 to its transcriptional activity raises the interesting issue of additional STAT3 post-translational modifications. STAT3 has now been reported to be tyrosine, serine and threonine phosphorylated [10], acetylated [11], alkylated [12], methylated [13], ubiquitinated [14] and glutathionylated [15]. However, it is important to note that all of these modifications were identified using STAT3 protein purified from entire cells - therefore no knowledge of compartment specific modifications and/or combinations of modifications that may be critical to STAT3 activity is available. Additionally, all of these modifications were exclusively tested for their capacity to modulate transcriptional activity and as such their influence on other non-nuclear activities of STAT3 remains unknown.

2.2 Mitochondrial STAT3

The linear signal transduction pathway described in section 1 is too simplistic. Early evidence for alternate and non-nuclear activities of STAT3 came from the identification of higher order STAT protein complexes ranging between 200kDa and 1MDa [16-18]. In addition STAT3 associates with other cytosolic structures including focal adhesions, microtubules, and the mitotic spindle, as well as with membrane elements, such as plasma membrane rafts [19], endo-lysosomes [20] and mitochondrial enriched fractions [21-23].

Recently STAT3 was shown to be present in a protease-resistant mitochondrial fraction of cardio-myocytes, pro-B cells and in Ras-transformed cell lines demonstrating its capacity to be imported into the mitochondria [21, 22]. This pool of STAT3 supports the activity of the Electron Transport Chain (ETC) required for ATP production and the opening of the mitochondrial permeability transition (mPTP) pore. These mitochondrial activities of STAT3 have consequences both in normal tissue homeostasis (e.g., neurite outgrowth and cardiac function) and in pathologic conditions (e.g., tumor growth and tissue damage in response to ischemia/reperfusion injury) discussed in section 2.2.3.

The study of the mitochondrial activities of STAT3 is an exciting new field that is likely to contribute much to our knowledge of how STAT3 regulates many biological processes both in normal and in diseased tissues. However, the discovery of this pool of STAT3 poses more questions than it answers including the mode of import and mechanism of action, both of which are addressed in sections 2.2.1 and 2.2.2.

2.2.1 STAT3 mitochondrial import

Mitochondria are a critical organelle that are the major source of cellular ATP, but are also critical for the synthesis of ribose, lipid and protein necessary for cellular replication. They retain their own genome that encodes a mere 13 of the more than 1,000 known mitochondrial proteins. Therefore, over 98% of mitochondrial proteins need to be imported following synthesis on cytosolic ribosomes highlighting the need for an efficient and accurate mechanism for targeting nuclear encoded proteins to the correct sub-mitochondrial compartment [24]. Mitochondrially directed proteins contain targeting motifs that are recognized by receptors of the Transporter Outer Membrane (TOM) complex. Most mitochondrial proteins carry an N-terminal pre-

sequence that is recognized by TOM receptors TOM20 and TOM22 (Fig. 1). Following receptor binding proteins are shuttled through the TOM40 outer membrane pore and into the inner membrane space. However, nearly a third of imported mitochondrial proteins lack this pre-sequence [25] and are directed to the mitochondria by a cryptic internal targeting signal. STAT3 mitochondrial import is enhanced by PKC-dependent phosphorylation and interaction with the HSP70 and HSP90 chaperones [26]. This is consistent with TOM70 recognition and import, in which proteins are delivered to the import machinery by chaperones and shuttled through the outer membrane. Our unpublished data show that deletion of the STAT3 amino terminal 134 amino acids does not alter mitochondrial import. Therefore, despite the capacity of STAT3 to interact with TOM20 [27, 28], it is likely that STAT3 is imported through a TOM70 dependent mechanism although this is still to be confirmed (Fig. 1). Interestingly, sub-mitochondrial fractionation experiments suggest that STAT3 associates with the inner mitochondrial membrane or is found in the mitochondrial matrix [28]. This implies that STAT3 also needs to be shuttled across the mitochondrial inner membrane which requires interaction with the mitochondrial Transporter Inner-Membrane (TIM) import complex machinery although there is currently no report of the mechanism of STAT3's movement across the inner membrane.

Recently, Tamminerni *et. al.* used *in vitro* import of radio-labelled STAT3 into isolated mitochondria to show that mitochondrial import was dependent on phosphorylation on S727 [27]. In contrast, when we isolated mitochondria from the liver and lymphocytes of STAT3 S727A knock-in mice we observe no deficit in the mitochondrial pool of STAT3 [29] (Fig 1). These mice are homozygous for S727A mutant STAT3 and STAT3 is driven off the endogenous promoter. These apparently contradictory pieces of data could be reconciled if the loss of STAT3 S727 phosphorylation altered the kinetics of import resulting in less mitochondrial STAT3 in transient *in vitro* experiments, but would not alter the steady state pool of mitochondrial STAT3 *in vivo*. The identification of the STAT3 mitochondrial import signal, and therefore the capacity to block STAT3 mitochondrial import, remain one of the most important discoveries in this emerging field.

2.2.2 STAT3 mitochondrial functions

Loss of STAT3 reduces the activity of the ETC, especially complex I, II and V [21, 22] which can be restored by reconstituting these cells with a mitochondrially restricted form of STAT3 (Fig. 2). Subsequent studies confirmed these findings and have additionally shown that STAT3 affects the activity of complex III and IV of the ETC [30-32]. STAT3-dependent activity of complex I and II is dependent on the phosphorylation of S727 [21, 22], and to a lesser extent an intact DNA binding domain [32, 33]. STAT3 immunoprecipitates with an antibody cocktail that recognizes complex I [22] and is well documented to interact with GRIM-19, an integral component of complex I [27, 34, 35], which could indicate a direct regulation of complex I through physical interaction. However, this would not explain the effect of STAT3 loss on the activity of other ETC complexes. Moreover, mass-spectrometry has shown that there is a substantially lower concentration of STAT3 than ETC proteins [36] and a stoichiometry approaching 1:1 would be expected for a change in activity due to direct interaction. Therefore, whilst STAT3 does impact ETC activity, no mechanism has been described yet.

The mitochondrial permeability transition pore (mPTP) enables passage of solutes of up to 1.5 kDa across the mitochondrial inner membrane in response to calcium overload, oxidative stress and membrane depolarization. Whilst the mPTP is important to normal mitochondrial homeostasis it is predominately studied in the context of cell death. Prolonged opening of the mPTP leads to the tissue death observed following ischemia/reperfusion injury. Whilst incompletely described, the mPTP is dependent on cyclophilin D, which can be inhibited by cyclosporine A (for review see [37]). STAT3 directly interacts with cyclophilin D and STAT3 knockout cardiomyocytes require lower ADP and calcium concentrations to induce mPTP opening [28]. Additionally the use of the STAT3 inhibitor stattic on isolated mitochondria reduced the calcium and ADP concentrations required to trigger mPTP opening [28, 38]. These data implicate STAT3 as a repressor of mPTP opening, which is likely to contribute to both the response to ischemia/reperfusion and may lead to delayed cell death in cancer (Fig. 2).

Mitochondria have a discrete circular, double stranded genome that encodes 13 ETC proteins, 22 tRNAs and 2 rRNAs, which is compacted into nucleoprotein complexes termed nucleoids [39]. STAT3 closely associates with ATAD3 [40], a protein involved in nucleoid assembly and organisation through its recruitment to the regulatory D-loop of the mitochondrial genome [41].

Transcription of mitochondrial (mt)DNA starts at one of three promoter regions (H1, H2 and L) within the D-loop and generates polycistronic mRNA transcripts, which are cleaved to generate mature mRNA, tRNA and rRNA. Transcription of mtDNA is largely controlled by three nuclear encoded proteins: the monomeric RNA polymerase (RNAP) or POLRMT in humans, mitochondrial transcription factor A (TFAM), and mitochondrial transcription factor B1 and B2 (TFB1M, TFB2M). STAT3 immunoprecipitates with TFAM [42] and chromatin immunoprecipitation shows that STAT3 binds to mtDNA at both the regulatory D-loop of platelets [23] and at consensus STAT binding sites throughout the mitochondrial genome of keratinocytes [42]. Deletion of STAT3 from keratinocytes results in an increase in the expression of mtDNA encoded genes implying that STAT3 represses transcription of the mitochondrial genome (Fig. 2). The capacity of STAT3 to regulate transcription of mtDNA encoded genes is a potential mechanism whereby a relatively low concentration of protein could regulate the activity of more abundant protein complexes. However it should be noted that whilst STAT3 represses mtDNA transcription in keratinocytes and platelets it enhances the activity of the ETC in pre-B cells, cardiomyocytes and cancer cells which is counter-intuitive if these two activities are inter-dependent.

In summary STAT3 can alter the activity of the ETC, the opening of the mPTP and the transcription of mtDNA encoded genes (Fig.2), however as yet there is no clear mechanistic insight explaining STAT3 mitochondrial import, nor how it regulates these diverse mitochondrial activities. However, the biological significance of mitochondrial STAT3 mean that these are critical questions to address.

2.2.3 Biological roles of mitochondrial STAT3 in health and disease.

The capacity of STAT3 to facilitate malignant transformation has typically been attributed to its activity as a tyrosine phosphorylated transcription factor downstream of common oncogenes (e.g., JAK2, EGFR, Src, NPM-ALK and elevated cytokine and growth factor secretion). Yet STAT3 was still shown to be critical for transformation by oncogenic mutation in the Ras GTPases in the absence of tyrosine phosphorylation [21], which is consistent with a non-canonical requirement for STAT3 in Ras-transformation. Indeed, STAT3 mutants that are transcriptionally inert (DNA binding domain, Y705F, SH2 domain and nuclear localization

mutants) efficiently supported Ras-transformation. The only residue that was shown to be required for transformation by Ras-oncogenes was phosphorylation on S727. Artificial targeting of STAT3 to the mitochondria in cells that were otherwise STAT3-deficient completely restored the capacity of Ras to transform these cells *in vitro* and *in vivo* which was attributed to the capacity of STAT3 to augment ETC activity and ATP production [21] (discussed in section 2.2.2). The importance of S727 phosphorylated mitochondrial STAT3 was recently confirmed *in vivo* in a KRas-driven Juvenile Myelomonocytic Leukemia mouse model [29]. The mitochondrial pool of STAT3 has now been linked to breast cancer [43], Barrett's esophagus [44], pancreatic cancer [45] and chronic lymphoid leukemia [46]. However, it is important to note that in many of these studies the requirement for the mitochondrial pool of STAT3 in a given tumor type was inferred from accumulation of STAT3 in the mitochondria, the use of mitochondrial targeted inhibitors or by using mitochondrially-targeted STAT3 mutants in the context of endogenous wild-type STAT3. It is critical to confirm the importance of mitochondrial STAT3 in cells that are deficient in the nuclear activities of STAT3 given the potency of the transcriptional activity of STAT3 to support a multitude of tumors.

The original description of a mitochondrial activity of STAT3 was in cardiomyocytes where it regulates ETC activity [22]. The reperfusion of ischemic tissue leads to drastic mitochondrial changes including increased reactive oxygen species (ROS) generated by ETC dysfunction, increased mitochondrial membrane potential, extended opening of the mPTP and ultimately tissue destruction. Blocking the activity of complex I of the ETC prior to reperfusion leads to a decrease in infarct size [47]. Fascinatingly, the presence of STAT3 in cardiac tissue is critical for pharmacological intervention and pre-conditioning to reduce infarct size [48], although this was not attributed directly to the mitochondrial pool of STAT3. However, there is substantial emerging literature documenting mitochondrial STAT3 is linked to processes critical in the response to ischemia/reperfusion such as ETC activity, production of ROS, membrane potential and opening of the mPTP. STAT3 knockout cardiomyocytes tolerate lower Calcium concentrations before the mPTP opening is triggered [28]. Recently mice were generated in which STAT3 is exclusively targeted to the mitochondria which has enabled more conclusive studies on the role of mitochondrial STAT3 in ischemia/reperfusion injury. This mouse model expresses a mitochondrially targeted STAT3 mutant that is incapable of binding to DNA

(E434A/E435A) under control of a cardiac specific promoter. These animals were seen to have slightly decreased ETC activity, produce less ROS and generate less tissue damage in response to ischemia/reperfusion injury [32, 33]. Together these data suggest that the mitochondrial pool of STAT3 alters the response to ischemia/reperfusion. However, there are several caveats to these studies. These mice still express endogenous STAT3 and therefore the effect of the mitochondrial pool of STAT3 cannot be distinguished from that of the total pool of STAT3. Additionally, the mitochondrial STAT3 expressed in these mice is defective for DNA binding. What effect this has on regulation of the expression of genes encoded by the mitochondrial DNA has not been defined. It would be intriguing to repeat these studies in a mouse that expresses a mitochondrially localized STAT3 containing an intact DBD and that lack the endogenous STAT3 gene in the heart.

3. Metabolic activity of STAT3, co-operation of nuclear and mitochondrial activities

The mitochondrial activity of STAT3 should not be considered in isolation. The production of ROS, ribose, ATP and other key biomolecules are critical to the cell and organism as a whole. STAT3-mediated nuclear gene transcription alters mitochondrial function and conversely the mitochondrial function of STAT3 influences the cellular ROS concentration, both of which are required for neoplastic transformation. A constitutive transcriptionally active STAT3 mutant (STAT3C), capable of transforming mouse embryonic fibroblasts [49] drives a metabolic shift common in many cancers – the Warburg effect. The Warburg effect describes a phenomenon in which tumors favor glycolysis (a cytosolic pathway) over oxidative phosphorylation (mitochondrial) to generate ATP [50]. Glycolysis is less efficient at converting glucose to ATP, but generates more biomolecules required for rapid proliferation. In the context of constitutively active STAT3 this metabolic shift is due to its nuclear activities. STAT3C induces the expression of a key regulator of metabolism Hypoxia Induced Factor 1 α (HIF1 α). Increased HIF1 α expression drives an increase in glycolysis. Interestingly, STAT3C expressing cells also have a decrease in mitochondrial activity due to a decrease in the expression of key nuclear encoded mitochondrial genes which are regulated in a HIF1 α – independent manner [51]. This is in contrast to observations in Ras-driven tumors in which a S727 phosphorylated, mitochondrial pool of STAT3 increases ETC activity. However, the STAT3C model is dependent on

augmented canonical, Y705 phosphorylated and transcriptionally active STAT3 which is required for transformation by mutant tyrosine kinases (e.g. JAK or EGFR) or sustained inflammation (e.g. IL-11 or IL-6). It remains to be seen whether other tumors that are dependent on the canonical, nuclear activity of STAT3 show a Warburg shift. It is possible that tumors expressing Y705 phosphorylated STAT3 will be dependent on the nuclear activities of STAT3 which will drive a Warburg shift. In contrast, tumors in which STAT3 is S727 phosphorylated in the absence of Y705 phosphorylation will display augmented mitochondrial ETC activity. However this remains to be tested.

The mitochondrial activity of STAT3 has additional consequences that are relevant to cancer. Mitochondrial accumulation of STAT3 correlates with altered concentrations of Reactive Oxygen Species (ROS) [34, 45, 52-54]. A mass-spectrometry based screen for mitochondrial-STAT3 dependent metabolites revealed that mitochondrial-STAT3 alters the glutathione (GSH) synthesis machinery [53]. One hypothesis is that mitochondrial STAT3 enhances the activity of the ETC resulting in increased production of ROS, triggering increased synthesis of GSH to scavenge ROS before it can reach detrimental concentrations (Fig. 2). This hypothesis is supported by data showing that pharmacological inhibition of GSH synthesis results in accumulated ROS, DNA damage and death in Ras-transformed, STAT3 expressing cell lines [53].

4. Conclusions

The biochemistry and biological consequences of JAK-STAT signaling has been described for over 2 decades. Yet novel aspects of these pathways are still being described. The mitochondrial pool of STAT3 is an emerging and exciting area of STAT biology. Through an unknown mechanism STAT3 traverses two mitochondrial membranes to reside in the mitochondrial inner membrane or the matrix where it augments the activity of the electron transport chain and impedes the opening of the mitochondrial permeability transition pore. This has consequences to cellular ATP production, ROS concentration, calcium homeostasis, and cell survival. Whilst mitochondrial STAT3 has been observed in every tissue examined its activities are best described in pathological conditions including cancer and ischemia/reperfusion. The

mitochondrial pool of STAT3 provides an ideal therapeutic target as the post-translational modifications that endow STAT3 with activity in the mitochondria are distinct from those in the nucleus. Therefore, specifically altering the activity of the mitochondrial pool of STAT3 will impact on disease states whilst preserving the critical nuclear activities of STAT3 that are necessary for normal tissue homeostasis. However, to achieve this, fundamental questions need to be answered including how STAT3 is targeted to the mitochondria, what it interacts with once imported and to define the post-translational modifications that control its activities.

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Figure Legends

Figure 1: Mitochondrial import of STAT3.

Mitochondrial proteins encoded by the nuclear genome are imported into the mitochondria via two major mechanisms. Proteins with an N-terminal targeting signal are typically recognized by the TOM20 receptor on the mitochondrial outer membrane. In contrast proteins with an internal targeting signal are recognized by TOM70, both classes of proteins are imported through a pore comprised of TOM40. There is some controversy about the requirement for STAT3 to be phosphorylated on S727 prior to mitochondrial import. Also whilst STAT3 can interact with TOM20, the amino terminus of STAT3 is not required for import. Therefore it is likely that STAT3 is imported via an internal targeting signal and TOM70 mediated import. Biochemical fractionation and functional studies imply that mitochondrial STAT3 localizes to the mitochondrial inner membrane or matrix space, implying that it must also engage the TIM complex that is required for transit across the mitochondrial inner membrane. However at this stage no information about a STAT3-TIM interaction is available.

Figure 2: Mitochondrial activity of STAT3

The activity of the mitochondrial pool of STAT3 is dependent on phosphorylation on S727, but independent of phosphorylation on Y705. The diverse mitochondrial activities of STAT3 include the regulation of the activity of the electron transport chain (ETC), the opening of the mitochondrial permeability transition pore (mPTP), and transcription of the mitochondrial genome (mtDNA). At least part of this activity is due to the interaction between STAT3 and complex I of the ETC, cyclophilin D (CypD), which is required for the opening of the mPTP and with key mtDNA transcription factors. The capacity of STAT3 to influence these key mitochondrial processes alter production of ATP, ROS and contribute to cellular transformation and death. However, as yet the mechanism by which a small pool of STAT3 can control such abundant protein complexes has not been elucidated.

Figure 1

Figure 2